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Mutational Analysis of GNAS1 in Patients with Pseudohypoparathyroidism: Identification of Two Novel Mutations*

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ABSTRACT

Pseudohypoparathyroidism (PHP) refers to two major variants that generally coexist in the same family, PHP type Ia (PHP Ia), in which both PTH resistance and a constellation of physical features, termed Albright's hereditary osteodystrophy (AHO), are present, and pseudopseudohypoparathyroidism (PPHP), in which AHO occurs without PTH resistance. Most patients with PHP Ia show a partial deficiency (50%) of G_s activity, due to loss of function mutations in $G_s\alpha$ gene (GNAS1). The present study reports clinical, biochemical, and molecular data of 8 unrelated families with PHP Ia and PPHP. The 13 exons of GNAS1 were screened for mutations by PCR and direct sequencing of the amplified products. We detected heterozygous mutations in the affected members of the 4 families in which PHP Ia was present. In 2 families 2 previously reported deletions in exons 5 and 7 were found, whereas in the other 2 families, 2 novel frameshift deletions were identified in exons 1 and 11, causing a premature stop codon in the mutant allele. No mutation was detected in the families in which PPHP was the only clinical manifestation.

In conclusion, we report the first mutational analysis of Italian patients with PHP Ia and PPHP, and we describe two novel deletions in GNAS1. Furthermore, we confirm that these mutations cannot be detected in families with isolated PPHP, suggesting that these forms of AHO are genetically distinct from PHP Ia. (*J Clin Endocrinol Metab* **85:** 4243–4248, 2000)

PSEUDOHYPOPARATHYROIDISM (PHP) refers to a heterogeneous group of rare metabolic disorders characterized by hypocalcemia and hyperphosphatemia due to PTH resistance associated with a constellation of physical features, including short stature, obesity, round face, brachydactyly, and sc calcifications, termed Albright's hereditary osteodystrophy (AHO) (1). The major variants, inherited as autosomal dominant disorders, include PHP type Ia (PHP Ia), in which both PTH resistance and AHO features are present; pseudopseudohypoparathyroidism (PPHP), in which AHO occurs in the absence of PTH resistance and which generally coexists with PHP Ia in the same family; and PHP type Ib, in which resistance to PTH is not associated with any somatic feature of AHO. In addition to resistance to PTH, patients with PHP Ia may have resistance to other hormones, such as TSH and gonadotropins, that stimulate cAMP formation in the target cells by interacting with receptors coupled to G_s, the stimulatory protein of adenylyl cyclase. Most patients with PHP Ia show a partial deficiency (50%) of G_s activity (2–4) due to a reduction in its α-subunit ($G_s\alpha$) messenger ribonucleic acid (mRNA) and protein. The human $G_s\alpha$ gene (GNAS1) contains 13 exons encoding the $G_s\alpha$ and is located at 20q13.11. Heterozygous loss of function mutations in GNAS1 have been identified in the majority of patients with PHP Ia and in their relatives affected with PPHP (5–19), whereas there is a general agreement that PHP Ib is not associated with this defect. GNAS1 mutations have been localized in the entire coding sequence of the gene; only a mutational hot spot has been identified to date, and it maps within exon 7 (7, 19–21), involving 35% of all mutations described. However, not all patients with PHP Ia and PPHP were found to carry detectable GNAS1 mutations (19, 22).

Many features of AHO are quite nonspecific or are present in other disorders, some of which are ascribed to specific chromosomal defects, such as the small terminal deletions on chromosome 2 in the AHO-like syndrome (23, 24). This makes the diagnosis of PPHP in families in whom PHP Ia is not present particularly difficult. Therefore, it has been proposed that the coexistence of multiple AHO manifestations probably increases the likelihood of a correct diagnosis of PPHP (22).

Here we report clinical, biochemical, and molecular analysis of eight unrelated families affected with AHO features associated or not with PHP Ia. In the four families in whom PHP Ia was present, heterozygous GNAS1 mutations were detected; in two of them we identified two novel frameshift deletions. No mutations were found in any of the families in whom PPHP was the only clinical manifestation.

Subjects and Methods

Patients

The study includes the families of eight probands: four affected with PHP Ia, three with PPHP, and one in whom the diagnosis of PHP Ia *vs.*

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PPHP is still uncertain. In PHP Ia patients the diagnosis was based upon the occurrence of PTH resistance (*e.g.* hypocalcemia, hyperphosphatemia, and raised serum PTH levels) together with AHO manifestations. In all PHP Ia patients hypothyroidism due to resistance to TSH (documented by raised serum TSH levels with an exaggerated response to TRH in the absence of antithyroid antibodies and in the presence of a normal thyroid scan) and requiring thyroid hormone replacement therapy was present. Hypogonadism was present in only 1 PHP Ia patient (A II 1), but it should be considered that two of six PHP Ia patients were of prepuberal age. All PPHP patients had several AHO features in association, including short stature, obesity, round face, brachydactyly, and sc calcifications without evidence of PTH resistance (Table 1).

In one patient (E II 1, Table 1) the diagnosis of PHP Ia *vs.* PPHP is still uncertain; she was first referred to our hospital at the age of 11 yr for growth retardation. At that time she presented short stature, round face, obesity, brachydactyly, sc calcifications, and only partial PTH resistance (high PTH levels with normal calcium and phosphate levels) with normal thyroid and gonadal function. The Ellsworth-Howard test, which would have been helpful in confirming the diagnosis, was not performed because of the unavailability of the human PTH-(1–34) synthetic peptide analog. Considering the families of PHP Ia probands (families A–D), PHP Ia was the only clinical manifestation in two families (A and B); in one (C) both PHP Ia and PPHP were present; and one case (D) was sporadic. Probands with PPHP belonged to families in whom familial (F) or sporadic (G and H) PPHP was the only clinical manifestation. In proband F III 1, who has inherited the disease from the mother, the diagnosis of PHP Ia cannot be ruled out, as she may manifest the hormonal resistance at a later age; this seems unlikely, however, as resistance, even in its mild forms, usually manifests within the first decade of life. Clinical and biochemical details for patients with PHP Ia and PPHP and their relatives are given in Table 1. Informed consent was obtained from all subjects involved in the study. The study was approved by the local ethical committee.

Methods

Genomic DNA was extracted by the phenol-chloroform method from peripheral blood leukocytes (Nucleon, Amersham Pharmacia Biotech, Aylesbury, UK). The $G_s\alpha$ gene (exons 1–13) (25) was then amplified by the PCR using the specific primers pairs shown in Table 2. Amplification of exons 2–12 included each bordering intron region, whereas for exon 1, because of the abundance of guanine and cytosine in the bordering regions, a DNA fragment from 20 bp downstream of the initiation codon to the donor site of intron 1 was amplified (22). A 50-μL reaction mixture [0.5–1 μg DNA sample, 50 nmol/L KCl, 50 nmol/L Tris-KCl (pH 8.3), 1–2 nmol/L $MgCl_2$, 40 pmol of each primer, and 2.5 U *Taq* DNA polymerase-AmpliTaq (Perkin-Elmer Corp., Foster City, CA)] was subjected to denaturation at 94 C for 3 min, followed by 34 cycles of 94 C for 45 s, at specific annealing temperature (Table 2) for 45 s, and 72 C for 45 s. A final cycle at 72 C for 10 min was carried out to allow complete extension of the amplified fragments. The amplified products were then visualized on a 3% agarose gel stained with ethidium bromide. Both strands of each exon were finally sequenced for each patient; sequencing of the PCR products using both sense and antisense primers was performed using the AmpliTaq BigDye Terminator kit and 310 Genetic Analyzer (Perkin-Elmer Corp., PE Applied Biosystems). Whenever a mutation was detected, it was confirmed by performing a new genomic DNA extraction and subsequent sequencing analysis.

Total RNA was extracted from peripheral blood from patient H II 1, and the levels of $G_s\alpha$ RNA transcripts were evaluated by semiquantitative RT-PCR using an appropriately selected primer pair (5′-AGCACCATTGTGAAGCAGATG-3′ and 5′-TGCTTGTTGAGGAAACAGGAT-3′, spanning exons 2–11, with an annealing temperature of 58 C) and compared with those from three normal subjects; the hypoxanthine-guanine phosphoribosyltransferase gene was used as an internal standard. For each complementary DNA, preliminary experiments were conducted to determine the PCR cycles corresponding to the exponential phase, as previously described (26). PCR products were visualized on a 2% Nusieve-1% agarose gel, and the bands were evaluated by an imaging densitometer (GS-700, Bio-Rad Laboratories, Inc. Richmond, CA).

Standard chromosomal analysis on peripheral lymphocytes from patient H II 1 was performed.

Results

Direct sequencing of the amplified genomic DNA fragments revealed heterozygous frameshift mutations in all patients affected with PHP Ia. Of the four mutations, two were novel deletion mutations in GNAS1. In particular, patient A II 1 showed a novel heterozygous 2-bp deletion within codon 287 in exon 11, which results in a premature stop codon in position 298 (Fig. 1). The same deletion was found in the patient's mother, who presented the same clinical and biochemical features. No other family member was available for study. The other novel mutation was a heterozygous 1-bp deletion within exon 1 (codon 38), which results in a premature stop codon in position 57 (Fig. 2). This mutation was found in patient C II 1, who was affected with PPH Ia, and in the patient's mother, who was affected with PPHP. No mutation was detected in the unaffected father and brother.

The other two GNAS1 mutations have been reported previously (7, 14). In proband B II 1 and her brother, both affected with PHP Ia, a heterozygous 1-bp deletion in exon 5 (Pro^{116}) involving a cytosine residue and determining a premature stop codon in position 132 was detected. Their parents were not available for biochemical and genetic assessment. Sequence of DNA from patient D II 1 revealed a previously described 4-bp deletion in exon 7 involving codons 189 and 190 that determines a premature stop at codon 202 (7). Both parents had normal sequences, thus indicating that a *de novo* mutation had occurred in this girl.

No mutations could be found in any of the PPHP patients without relatives with PHP Ia (families F, G, and H) or in the patient with uncertain diagnosis (E II 1). It is worth noting that all of these patients showed the typical constellation of physical features, including short stature, obesity, round face, brachydactyly, and sc calcifications, that were superimposable to those observed in PPHP patients with GNAS1 mutations (Table 1). In the familial case (F), these features were present in the proband, her mother, her maternal aunt, and her grandfather (Table 1). Semiquantitative RT-PCR from peripheral lymphocytes of patient H II 1 showed levels of $G_s\alpha$ RNA comparable to those found in controls (OD target/standard ratio, 0.63 ± 0.09 in controls *vs.* 0.70 in the patient).

In patient H II 1 chromosomal analysis on peripheral lymphocytes was also performed. The chromosomal study revealed a normal female karyotype (46,XX), with no visible deletions on the long arm of chromosome 2.

A T→C transition in exon 5 within the codon corresponding to isoleucine at position 131 was detected in four of eight patients (C II 1, D II 1, F III 1, and G II 1), thus confirming the high prevalence of this polymorphism (47%) in the population (8).

Discussion

Here we describe the first mutational analysis of GNAS1 in Italian patients affected with pseudohypoparathyroidism. Our series included families in whom PHP Ia was in association, or not, with PPHP and families in whom PPHP was the only clinical manifestation. By analyzing the entire GNAS1-coding sequence, we detected heterozygous mutations in the totality of patients affected with PHP Ia and their

TABLE 1. Clinical, biochemical, and molecular data of patients with PHP Ia and PPHP and their relatives

Patient no.	Sex	Age (yr)	Diagnosis	Clinical signs	Imaging	Ca (mmol/L; normal, 2.2–2.6)	P (mmol/L; normal, 0.8–1.5)	PTH (pmol/L; normal, 1.1–6.8)	TSH (mU/L; normal, 0.3–4.2)	GNAS1 mutations
A I 1	F	49	PHP Ia	Br; Ob; SC; RF; SS	IC; SM	1.58	1.69	21.9	4.7	Exon 11, 2-bp del
A II 1[a]	F	20	PHP Ia	Br; Ob; SC; RF; SS	IC; SM	1.59	2.32	8.7	8.2	Exon 11, 2-bp del
B II I[a]	F	27	PHP Ia	Br; Ob; SC; RF; SS	SM	1.58	2.31	19.8	9.6	Exon 5, 1-bp del
B II 2	M	20	PHP Ia	Br; Ob; SC; RF; SS	SM	1.63	2.15	25.0	11.5	Exon 5, 1-bp del
C I 1	F	32	PPHP	Br; Ob; SC; RF; SS	SM	2.35	0.95	3.7	2.2	Exon 1, 1-bp del
C I 2	M	35	N	N		2.4	1.15	4.8	1.5	WT
C II 1[a]	F	7	PHP Ia	Br; Ob; RF; SS	SM	2.5	1.7	34.6	12.6	Exon 1, 1-bp del
C II 2	M	11	N	N		2.25	1.2	5.1	3.2	WT
D I 2	M	34	N	N		2.3	0.9	1.9	0.6	WT
D I 1	F	30	N	N		2.5	1.4	4.5	2.1	WT
D II 1[a]	F	6	PHP Ia	Br; Ob; SC; RF; SS	SM	2.04	2.4	26.3	5.4	Exon 7, 4-bp del
E I 1	F	45	N	N		2.45	1.22	6.1	1.9	Not tested
E I 2	M	47	N	N		2.3	1.1	2.8	1.0	Not tested
E II 1[a]	F	16	PHP Ia/PPHP	Br; Ob; SC; RF; SS	SM	2.22	0.99	13.3	2.17	WT
F I 2	M	71	PPHP	Br; Ob; RF; SS		2.3	0.9	4.2	1.6	Not Tested
F II 1	F	45	PPHP	Br; Ob; SC; RF; SS	SM	2.35	1.0	4.9	3.3	Not Tested
F II 3	F	40	PPHP	Br; Ob; RF; SS		2.4	1.3	5.5	0.9	Not Tested
F III 1[a]	F	13	PPHP	Br; Ob; SC; RF; SS	SM	2.35	1.4	2.7	2.7	WT
G I 1	F	29	N	N		2.4	1.1	3.5	2.6	Not tested
G I 2	M	30	N	N		2.5	0.9	4.7	0.5	Not tested
G II 1[a]	M	3	PPHP	Br; Ob; SC; RF; SS	SM	2.42	1.15	6.5	0.9	WT
H I 1	F	69	N	N		2.3	1.2	5.0	1.2	Not tested
H II 1[a]	F	40	PPHP	Br; SC; RF; SS	IC; SM	2.27	0.96	5.1	2.2	WT

Capital letters indicate the family; roman numbers indicate the generation.

[a] The proband; arabic numbers represent single subjects (1, mother; 2, father; 3, maternal aunt).

F, Female; M, male; WT, wild type; N, normal; Br, brachytactyl; Ob, obesity; SC, sc calcification; RF, round face; SS, short stature (<2 SD; IC, intracranic calcification; SM, shortening of metacarpals. The age refers to the present.

TABLE 2. Primers and annealing temperatures used to amplify GNAS1, exons 1–13

Exon	Upper primer	Lower primer	Annealing temperature (C)
1	5′-ATGGGCTGCCTCGGGAACAG-3′	5′-TTACCCAGCAGCAGCAGGCG-3′	66
2	5′-AAAATGCCTCCTTCATAACCTGA-3′	5′-GCCCACCTATACTTCCTAAAG-3′	56
3	5′-ATGGTTGAGGAATGTAGAGAGACTG-3′	5′-CAGTATGATCTTCATGTTTGTTTG-3′	56
4–5	5′-ATGAAAGCAGTACTCCTAACTGA-3′	5′-TGGATGCTCCTGCCCATGTG-3′	56
6	5′-ATTAGTTCAAGCTCTTGCCTTTC-3′	5′-TTGTCTGTTTTATGTGCTGATGG-3′	58
7	5′-TGCTGCATAACTGTGGGACG-3′	5′-GTAGTTTGGAAAGAGGGCTCAG-3′	58
8	5′-GTTGGCTTTGGTGAGATCCA-3′	5′-AGAAACCATGATCTCTGTTATA-3′	56
9	5′-ACAGAGATCATGGTTTCTTG-3′	5′-TTAACCAAAGAGAGCAAAGC-3′	54
10	5′-TGTTAGGGATCAGGGTCGCTG-3′	5′-AACAGTGCAGACCAGGGCCTCCTG-3′	66
11	5′-CAGGAGGCCCTGGTCTGCACTGTT-3′	5′-AGAACCACCGCAATGAACAGCC-3′	66
12	5′-AGACTTCAGGAGCTACAGAGA-3′	5′-AGAGGAGGAACAAGAGAGGAA-3′	56
13	5′-CATCAGAGGCTGGCTGACAGCG-3′	5′-AAGGCTTTAATTAAATTTGGG-3′	56

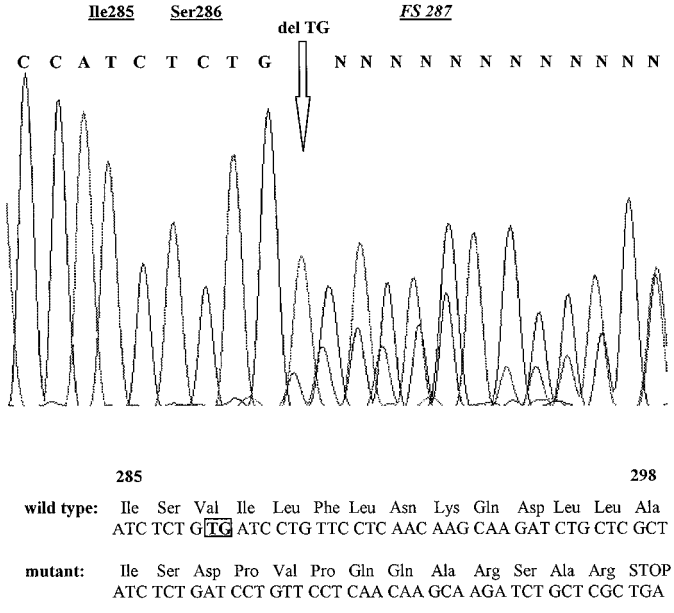

FIG. 1. Sequencing analysis of exon 11 in patient A II 1. The figure shows the 2-bp frameshift deletion at codon 287 that results in a premature STOP at codon 298. The same deletion was found in the proband's mother, who is also affected by PHP Ia.

relatives with PPHP. The high prevalence of GNAS1 mutations in our series may result from the screening method used, *i.e.* direct sequencing of the entire coding region of the gene, including exon 1, that is difficult to amplify due to the richness of guanosines and cytosines in its boundaries. Indeed, a prevalence of GNAS1 mutations of about 50% in patients with PHP Ia has been recently reported (19).

By direct DNA sequencing we identified two novel frameshift mutations within exons 1 and 11, thus expanding the spectrum of GNAS1 mutations associated with PHP1 and PPHP. The mutation in exon 1 (ΔR38) was a 1-bp deletion affecting codon 38 and resulting in a premature stop at codon 57, thus determining a truncated protein in the guanosine triphosphatase domain. This heterozygous alteration is predicted to be responsible for a reduced $G_s\alpha$ activity as demonstrated for the other previously reported deletions in exon 1 (5, 18). This novel mutation was associated with PHP Ia in the proband and with PPHP in the mother, providing further evidence that the same genetic defect may lead to two distinct presentations in PHP families. A normal GNAS1 sequence was detected in the father and brother, who were both unaffected. As frequently observed in PHP Ia patients, the two affected subjects showed resistance to other hormones acting through G_s-coupled receptors, in particular TSH. This mutation is the third mutation affecting exon 1, confirming that this exon is subject to undergoing genetic alterations (5, 18) and should therefore be included in the analysis of GNAS1 mutations.

The second novel mutation was a 2-bp deletion identified in exon 11, where no mutations have been reported to date. This frameshift (ΔV287) encodes a premature stop 11 codons downstream from the deletion and results in a severe alteration of the C-terminal region, lacking the G protein-coupled receptor interaction domain (encoded by exons 12 and 13) (25, 27). This mutant protein is thus likely to be functionally inactive. This mutation was detected in the proband and her mother, who were both affected by PHP Ia; the other family members were not available for biochemical and molecular analysis.

The pattern of transmission of these two novel heterozygous mutations is consistent with the general model proposed for AHO. Indeed, in our series all familial PHP Ia cases inherited the disease from the mother, in agreement with a possible role of paternal imprinting in the genesis of AHO. In fact, it has been proposed that when the mutated paternal allele is inherited, offspring will show a PPHP phenotype, whereas when the mutated maternal allele is inherited, clinical and biochemical characteristics of PHP Ia will be present (28, 29).

Of the two previously reported mutations, one was a heterozygous deletion in exon 5 affecting proline 116 (ΔP116) and introducing a stop codon 16 amino acids downstream. Also, this mutation has been shown to prevent the generation of a normal full-length $G_s\alpha$ protein, resulting in a partial deficiency (50%) of $G_s\alpha$ activity (14). This alteration, which is close to another known substitution ($Pro^{115}Ser$) (19), is predicted to disrupt the highly conserved domain of $G_s\alpha$ that interacts with adenylate cyclase (27). Thus, it seems that the particular region located in exon 5 and involving prolines 115 and 116 is subject to undergoing mutational changes with a significant frequency, and it could represent a new mutational hot spot in GNAS1. The last mutation described in this study (patient D II 1) is the well known deletion in exon 7 that in this family represented a *de novo* mutation, as it was detected in neither the mother nor the father. To date, this 4-bp

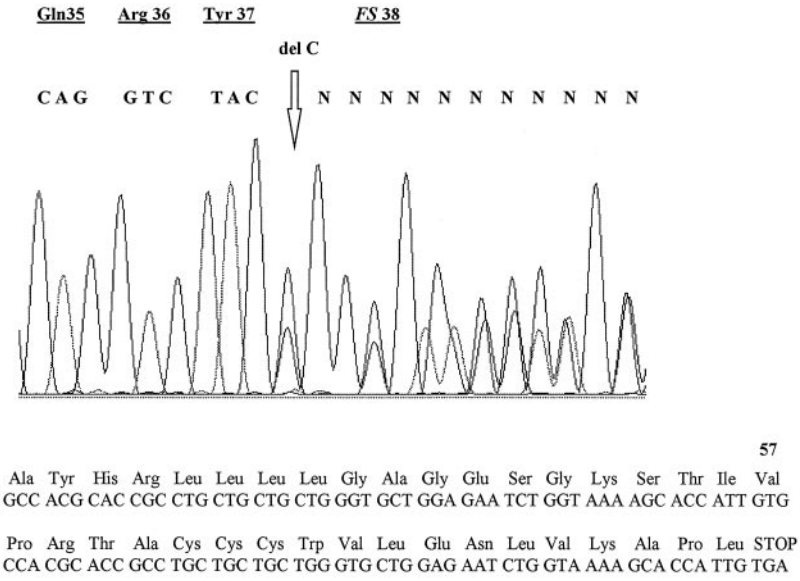

FIG. 2. Sequencing analysis of exon 1 in patient C II 1, showing a cytosine deletion at codon 38 and determining a STOP codon in position 57. The patient's mother, affected by PPHP, shows the same mutation, whereas normal sequences were seen in the patient's father and brother.

deletion in exon 7 represents the only defined deletional hot spot in GNAS1, involving a defined consensus sequence for the arrest of DNA polymerase α (30).

In the other families included in our analysis, in whom sporadic or familial PPHP was the only clinical manifestation, no alterations in the GNAS1-coding sequence were found, in agreement with previous reports (19, 22). Our results support the view that PHP Ia and isolated PPHP may represent two genetically distinct entities. Admittedly, the possibility that a defect might exist in the promoter region or in other regulatory intronic sequences of GNAS1 cannot be completely excluded. However, the finding of normal levels of $G_s\alpha$ mRNA in patient H II 1 does not support this hypothesis; semiquantitative RT-PCR was, in fact, performed using a primer pair that spanned exons 2–11, thus including almost the entire $G_s\alpha$ mRNA; functional regulatory regions are supposed to be necessary for the transcription of such product.

Furthermore, it should be considered that isolated PPHP is often difficult to distinguish from other heterogeneous disorders, such as the AHO-like syndrome or other skeletal defects, for which other chromosomal locations have been identified (23, 24, 31). However, in the PPHP patient (H II 1) in whom it was possible to perform chromosomal analysis on peripheral lymphocytes, no visible deletions on the long arm of chromosome 2, which have been reported to occur in a subset of patients with AHO-like disorders (23, 24), were present. Moreover, despite the difficulties in correctly defining subjects with AHO, the patients included in our study were diagnosed with PPHP on the basis of strict criteria, *i.e.* coexistence of specific and aspecific features, including short stature, obesity, round face, brachydactyly, and sc calcifications, all actually indistinguishable from those present in patients with PHP Ia and PPHP carrying GNAS1 mutations.

In conclusion, we report here the first mutational analysis of Italian patients with PHP Ia and PPHP. By analyzing the entire GNAS1-coding sequence we detected heterozygous mutations in the totality of patients affected with PHP Ia and their relatives with PPHP and identified two novel frameshift deletions affecting exons 1 and 11. No genetic alterations were found in families with PPHP as the only clinical manifestation. Further studies will be necessary to characterize the molecular defects responsible for PPHP and to understand the relationship between this disorder and PHP Ia.

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